In vitro selection of mutants: Inducible gene regulation for salt tolerance

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Abstract. Regulation of differentially expressed genes in plants may be involved in inducing tolerance to stress. Isogenic salt-sensitive and salt-tolerant alfalfa lines were investigated for molecular differences in their response to salt. The genes, which are differentially induced by salt in the salt-tolerant alfalfa cells and are also regulated by salt at the whole plant level, were cloned. Both transcriptional and post-transcriptional mechanisms influenced salt-induced product accumulation in the salt-tolerant alfalfa. The salt-tolerant plants doubled proline concentration rapidly in roots, while salt-sensitive plants showed a delayed response. To understand the regulatory system in the salt-tolerant alfalfa, two genes that are expressed in roots were studied. *Alfin1* encodes a zinc-finger type putative DNA transcription factor conserved in alfalfa, rice and *Arabidopsis*, and *MsPRP2* encodes a protein that serves as a cell wall-membrane linker in roots. Recombinant *Alfin1* protein was selected, amplified, cloned and its consensus sequence was identified. The recombinant *Alfin1* also bound specifically to fragments of the *MsPRP2* promoter *in vitro*, containing the *Alfin1* binding consensus sequence. The results show unambiguously binding specificity of *Alfin1* DNA, supporting its role in gene regulation. *Alfin1* function was tested in transformed alfalfa *in vivo* by over-expressing *Alfin1* from 35S CaMV promoter. The transgenic plants appeared normal. However, plants harboring the anti-sense construct did not grow well in soil, indicating that *Alfin1* expression was essential. *Alfin1* over-expression in transgenic alfalfa led to enhanced levels of *MsPRP2* transcript accumulation, demonstrating that *Alfin1* functioned *in vivo* in gene regulation. Since *MsPRP2* gene is also induced by salt, it is likely that *Alfin1* is an important transcription factor for gene regulation in salt-tolerant alfalfa, and an excellent target for manipulation to improve salt tolerance.

1. INTRODUCTION

Salt affects approximately a third of the arable land in the world. As more land becomes saline through poor irrigation practices, the impact of salinity on crop production is becoming an increasingly serious problem world wide, and has created a pressing need to improve salt-tolerance in plants. Improvement for salinity and drought tolerance of crop plants by genetic means has been an important but largely unfulfilled aim of modern agriculture. Other approaches have focused on the selection for improved physiological characteristics and metabolic pathways [1, 2].

An alternate genetic strategy has been pursued for improving salinity tolerance by combining selection of salt-tolerant cells in culture, followed by regeneration of salt-tolerant plants, and identification and testing of genes important in conferring salt tolerance. While most of the research work was done on alfalfa, the selection method is also applicable in rice [3, 4]. The regulation of differentially expressed genes is vital during development and differentiation, hence, mutations in these processes have the potential to provide tolerance against plant stress. The hypothesis in selection for salt tolerance is that over-expression of genes for any physiological system that becomes limiting under salt stress is likely to provide increased salt tolerance in cells and the plant. Incremental improvement in salt tolerance could be obtained from enhanced expression of genes in different physiological systems [4, 5]. Therefore, isogenic salt-sensitive and salt-tolerant alfalfa cells and plants were used for molecular studies to

investigate the differences in their responses to salt. The multigenic differences in regulation of endogenous genes [6] are consistent with the concept that salt tolerance is a quantitative trait [7] and the induction of many genes during salt/drought stress of plants [8, 9]. Since the salt tolerance trait in the mutants studied is heritable [10, 11], the multigenic responses indicate that the mutation is likely in the regulatory system. This leads to changes in gene regulation, associated with the ability to survive and grow under otherwise lethal conditions.

Previous studies with rice [11] and alfalfa [12] have demonstrated that cellular salt-tolerance can provide protection at the whole plant level, and can be a useful tool in selection for tolerance. When successful, the cellular selection and regeneration approach relies on identification of mutants optimized for continued survival and productive growth under saline conditions. However, it does not provide ready identification of the genes involved, and requires additional information about altered regulation of the genes [13, 14]. Several genes have been cloned that are differentially induced by salt in cells of the salt-tolerant alfalfa and are also regulated by salt at the whole plant level. In the present study, the root and shoot response to salt stress was investigated for proline accumulation as a measure of osmo-protection in the tolerant phenotype. Both the transcriptional and post-transcriptional mechanisms that contribute to gene product accumulation in the tolerant alfalfa growing in salt were studied. To understand the changed regulatory system in alfalfa, two genes, *Alfin1* and *MsPRP* that are expressed in roots were studied. *Alfin1* encodes a zinc-finger type putative DNA transcription factor, conserved in alfalfa, rice and *Arabidopsis*. *MsPRP2* encodes a protein that might serve as a cell wall-membrane linker in roots. The function of *Alfin1* was established as a transcriptional regulator by *in vitro* binding of the recombinant protein to specific DNA sequences, including fragments of the *MsPRP2* promoter [15]. The transgenic alfalfa over-expressing *Alfin1* from the 35S cauliflower mosaic virus (CaMV) promoter have increased levels of *MsPRP2* transcripts; thus *Alfin1* functions also *in vivo* as a regulator in gene expression [16]. Hence, it was interesting to characterize the role of *Alfin1* in salt tolerance and general gene regulation, since *MsPRP2* is salt-inducible *in vivo*.

2. MATERIALS AND METHODS

Alfalfa, *Medicago sativa* L. cv. 'Regen S', salt-sensitive callus cultures, selection of salt-tolerant lines and regenerated salt-tolerant plants have been previously described [10, 17]. Selection of salt-tolerant rice lines from two elite US rice lines (L-202 and M-202) and regeneration and testing of plants from the salt-tolerant lines was done as described previously [11]. Samples for molecular analyses of all tissues were collected at the same time during day to avoid influences of the circadian rhythm. Standard molecular biology protocols were used [18].

Recombinant *Alfin1* was expressed in *Escherichia coli* BL21(DE3), using the PET-29b vector system (Novagen Inc.). The purified S-Tag-Alfin1 fusion protein was used in DNA binding assays. Purified protein was used in the "random DNA binding assay" to identify specific DNA binding sites recognized by the recombinant Alfin1. To select binding sites from a 67 bp DNA, a 25 bp degenerate sequence was flanked by a known sequence of DNA with restriction sites and the template for PCR primers. A single protein bound DNA band was isolated by gel retardation [19] and amplified by PCR. The bound sequence was enriched by sequential selection of *Alfin1* bound PCR amplified DNA. The final round of bound DNA was cloned, and the DNA binding sequence was determined as the consensus sequence represented in the insert of all individual clones that were able to bind recombinant *Alfin1*.

The gene construct for the over-expression and under-expression of *Alfin1* was developed using the *Alfin1* cDNA [12]. The coding sequence was placed downstream of the 35S cauliflower-mosaic virus (CaMV) promoter in the sense and anti-sense orientation. The constructs were transferred into a binary vector that also contained kanamycin-resistance gene, and transformation of alfalfa leaf-discs was accomplished with *Agrobacterium tumefaciens*. Transformed cells were selected on medium containing kanamycin, and transgenic plants were regenerated.

3. RESULTS AND DISCUSSION

3.1. Cellular selection and regeneration to obtain salt tolerant plants

The cellular selection and regeneration protocol was extended to rice to investigate if the protocol developed for alfalfa [10, 17] was applicable to other species. Cell lines tolerant to 1% (0.171 M) NaCl were obtained from calli initiated from salt-sensitive US elite rice lines L202 and M202, as well as indica rice varieties, 'Pokkali', 'IR28' and 'IR42'. Several plants were regenerated from salt-tolerant lines L-202, M-202 and 'Pokkali'. Seed was collected from four plants regenerated from the salt-tolerant L-202 line and two plants regenerated from the salt-tolerant 'Pokkali'. R_2 seedlings were germinated and tested for salt tolerance. Heritable improvement in salt tolerance was obtained in R_2 seedlings from one L-202 (R_4) plant that had been regenerated after 5 months selection on salt-containing medium. The salt tolerance of these seedlings was comparable to that obtained from 'Pokkali' under conditions where the unselected L-202 seedlings died [11]. The results indicated that improved cellular salt-tolerance could also provide increased salt-tolerance in rice at the whole plant level.

3.2. Salt dependent gene regulation in salt-tolerant alfalfa

The characterization of altered gene expression in salt-tolerant alfalfa by salt resulted in identification of a number of genes that are "up-regulated" by salt (Table I). Since, the salt-tolerant phenotype in alfalfa is a heritable semi-dominant trait [10], the altered regulation of many genes must be the result of a mutation in its regulatory pathway. The selected salt tolerant cell lines showed the interesting association of chloroplast activation and increased mRNA accumulation for photosynthesis related genes in the salt-tolerant phenotype. At least some of the mRNAs were translated into functional products. Detailed analysis, comparing steady state mRNA levels for salt induced genes in the tolerant alfalfa with nuclear and chloroplast "run-on" assays, was carried out to measure transcriptional activation. The analysis showed that both transcriptional and post-transcriptional regulation contributed to the increased levels of the gene products when the salt-tolerant cells are grown in salt [13]. It was also demonstrated that regulated tissue-specific expression overrode salt induction of mRNA accumulation for specific genes [20]. Interestingly, several of the genes cloned from callus appeared to function predominantly in the root (Table I).

The correlation of the osmoprotectant proline accumulation with salt-tolerance in the near-isogenic, salt-sensitive and salt-tolerant cell lines and the plants derived from them was investigated. Proline accumulation was studied under salt-stress for productive and non-productive growth in salt-tolerant and salt-sensitive alfalfa (Table II). Although, there was some variability in proline levels between the sensitive and tolerant plants, no consistent correlation could be drawn between high levels of proline in either roots or shoots of the salt-tolerant phenotype. However, both calli and roots [21] from salt-tolerant alfalfa accumulated proline very

rapidly on exposure to salt, while the response was delayed in roots of the salt-sensitive parent plant No. 1 (Table II), and of regenerated control plant, not selected on salt (data not shown). The rapid response in case of salt-tolerant alfalfa may have an early protective function, and apparently form a part of the multigenic response to salt-stress.

TABLE I. ACCUMULATION OF mRNA IN SALT-TOLERANT ALFALFA

Gene	Function[a]	Tissue specificity		
		Callus	Root	Shoot
Alfin1[b]	nuclear transcription factor	+	+	-
MsPRP2[b]	nuclear cell wall protein	+	+	-
pA18[b]	nuclear unknown	+	-	±
rbcS[c]	nuclear Rubisco, small subunit	+	+	nd
rbcL[c]	chloroplast Rubisco, large subunit	+	+	nd
cab1[c]	nuclear chlorophyll binding protein	+	nd*	nd
psbA[c]	chloroplast Q_B binding protein	+	+	nd
H3cI[d]	nuclear histone H3 (replic. dep.)	+	nd	nd
H3cII[d]	nuclear histone H3 (replic. ind.)	+	nd	nd
rps11[c]	chloroplast ribosomal protein 11	-	nd	nd
Msc27[d]	nuclear constitutive protein	-	-	-

*Not determined. [a]Function shown or implied by sequence similarity with proteins of known function.
[b]Expressed in callus and predominantly in roots. [c]Expressed in tolerant callus and predominantly in shoots.
[d]Expressed in callus, roots and shoots.

3.3. Two gene products for improved salt tolerance

A major focus of the study was on two novel genes from differential screening of alfalfa cDNA library from mRNA isolated from salt-tolerant cells grown for several months in 171 mM NaCl. One is *Alfin1*, which encodes a 28.8 kDa regulatory factor [12] with the putative zinc finger-binding domain (Fig.1). *Alfin1* transcription and mRNA accumulation is induced by salt in the salt-tolerant cells [13]. In plants, this mRNA is detected primarily in roots.

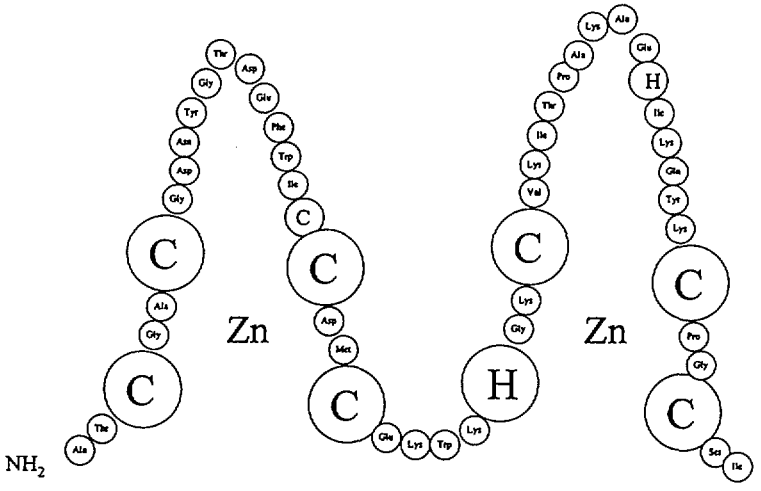
The other gene is *MsPRP2*, which encodes a proline-rich protein with a hydrophobic cysteine-rich carboxy terminus. This gene is also expressed predominantly in roots, and is specifically induced by long-term (i.e., one week) treatment with salt [22, 23]. The proline-rich amino terminal region of this protein has the characteristics of a cell wall component preceded by a cell wall targeting sequence, but the hydrophobic carboxy-terminus contains features characteristic of the nonspecific lipid transfer proteins. The bifunctional domains in *MsPRP2* could play a structural role as a wall-to membrane linker in plants, and thus contribute to structural integrity of the roots when exposed to salt.

The function of *Alfin1* as DNA-binding protein was investigated with the recombinant protein from *E. coli* [15] [Fig. 2] which was affinity purified, and used for selecting specific binding sequences from random DNA as described above.

TABLE II. PROLINE ACCUMULATION IN ALFALFA EXPOSED TO 171 mM NACL

Duration after NaCl exposure (hrs)	μg Proline/g fresh weight in plants			
	No. 1 (sensitive) Mean ± S.E.	No. 4T* Mean ± S.E.	No. 5T* Mean ± S.E.	No. 9T* Mean ± S.E.
Roots				
0	54 ± 44	103 ± 89	36 ± 15	47 ± 21
24	53 ± 29	170 ± 13	76 ± 13	91 ± 24
28	254 ± 168	430 ± 150	241 ± 173	336 ± 156
72	269 ± 27	513 ± 153	341 ± 181	271 ± 169
192	430 ± 118	675 ± 46	321 ± 86	182 ± 66
Shoots				
0	126 ± 63	102 ± 63	97 ± 35	119 ± 58
24	206 ± 5	353 ± 80	530 ± 292	472 ± 49
48	407 ± 188	706 ± 277	1289 ± 188	898 ± 82
72	1174 ± 15	790 ± 170	1381 ± 92	921 ± 130
192	1174 ± 80	1151 ± 256	1163 ± 49	1328 ± 311

*Tolerant type.



$C\text{-}X_2\text{-}C\text{-}X_{11}\text{-}C\text{-}C\text{-}X_2\text{-}C\text{-}X_4\text{-}H\text{-}X_2\text{-}C\text{-}X_9\text{-}H\text{-}X_5\text{-}C\text{-}X_2\text{-}C$

Fig. 1. Putative zinc finger-binding domain in the carboxy-terminus of Alfin1 protein as deduced from cDNA sequence (GenBank accession number L07291).

Sequence analysis of nine individually isolated clones revealed a consensus binding sequence for *Alfin1* which consisted of two to five triplet repeats of G rich sequences containing at least one GTG element. Both *Alfin1* and *MsPRP2* are expressed primarily in roots. This raised the question about the possible DNA binding sites for the *Alfin1* protein in the *MsPRP2* promoter. Three *TfiI* fragments were isolated from the *MsPRP2* genomic clone containing the promoter (Fig. 3), and reacted *in vitro* with purified recombinant *Alfin1*.

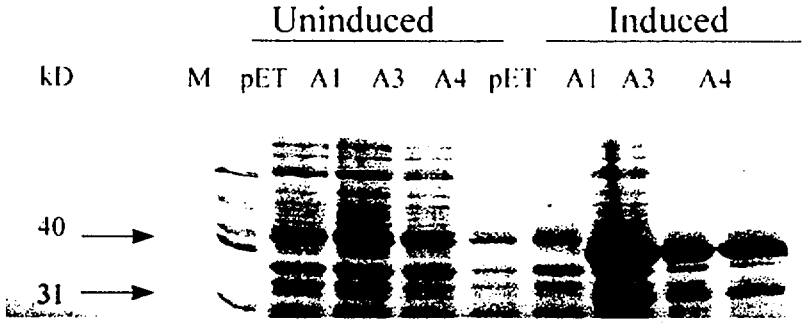


Fig. 2. Expression of Alfin1 fusion protein in E. coli. SDS-PAGE analysis of proteins expressed in crude extract from clones of E. coli transformed either with pET (vector) or A1, A3 and A4 (pET-Alfin1 construct). Lane 1- Molecular weight markers. Lanes 2,3,4 and 5- Protein from non-induced cells. Lanes 6,7,8 and - Protein from cells induced for 3 hr with IPTG.

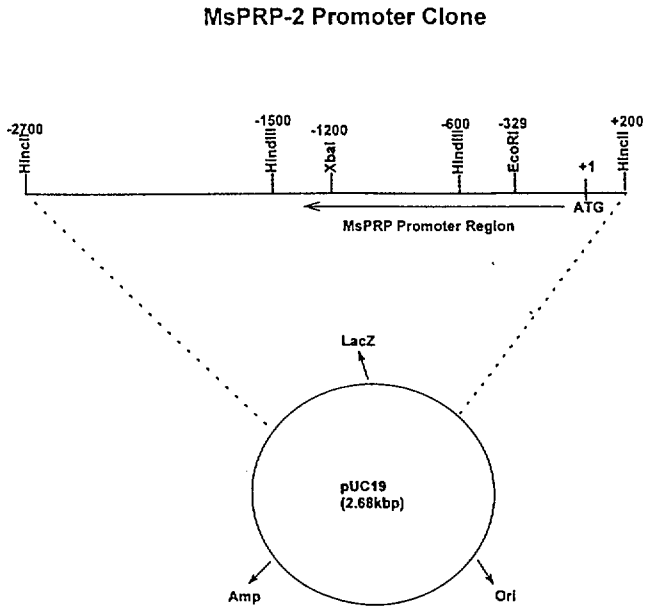


Fig. 3. Restriction map and organization of MsPRP2 promoter region in alfalfa.

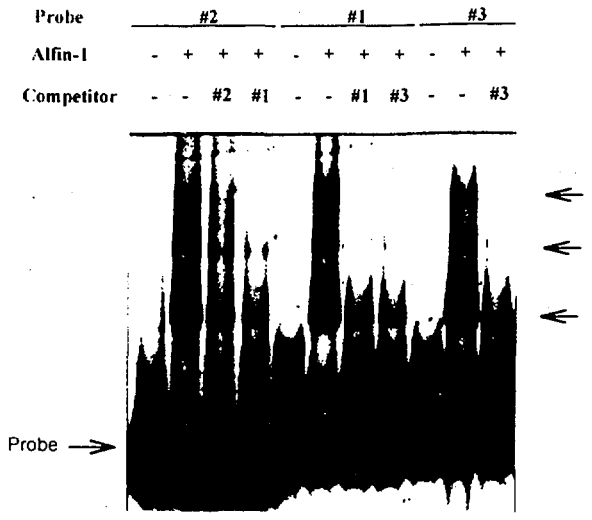


Fig. 4. DNA binding properties of Alfin1 fusion protein purified from E. coli. Gel retardation assay using 10 ng (20,000 cpm) of each ^{32}P labeled TfiI fragment of MsPRP2 promoter with equal amounts of recombinant Alfin1. Competition reactions were performed by pre-incubating with a ten fold excess (100ng) of each competitor fragment and subsequent addition of the ^{32}P labeled probe. The three contiguous TfiI fragments start at position -244 and extend to -864 bp as shown in Fig. 3. Probe: No. 1, 211 bp; No. 2, 187 bp; No. 3, 222 bp.2.

As shown in the gel retardation assay (Fig. 4), each of the *TfiI* fragments was able to bind recombinant *Alfin1* in a specific manner. This was demonstrated by fact that ten fold excess of the same fragment or one of the other fragments were able to compete the binding as shown by the diminished intensity of the retarded band(s). The sequence of each of the *TfiI* DNA fragments from *MsPRP2* was found to contain the *Alfin1* binding consensus sequence. A DNA fragment, which did not contain the *Alfin1* binding sequence, neither bound recombinant *Alfin1* nor was it able to act as a competitor for binding to each of the *MsPRP2* promoter fragments (data not shown). These results showed that *Alfin1* encodes a protein that bound DNA in a sequence specific manner *in vitro* and could act as a transcription factor with the *MsPRP2* gene as a potential endogenous *in vivo* target in alfalfa roots [15].

The *in vivo* function of *Alfin1* is currently being tested in transgenic alfalfa calli and in plants over-expressing *Alfin1* from constructs that transcribe *Alfin1* cDNA from the CaMV 35S promoter. The regenerated sense-plants appear normal despite the ubiquitous expression of *Alfin1* in both roots and shoots. Anti-sense plants develop poorly in soil, indicating that *Alfin1* expression is essential for growth and development. *Alfin1*over-expression in transgenic alfalfa leads to enhanced levels of *MsPRP2* transcript accumulation in callus and roots [16], indicating that *Alfin1* can act as a transcriptional activator for at least one salt inducible gene in alfalfa roots. The results suggest that *Alfin1* may be an important transcription factor involved in gene regulation in the salt-tolerant alfalfa. Future experiments will test the effect of *Alfin1* over-expression on other salt stress regulated genes, and investigate if *Alfin1* over-expression improves salt tolerance in alfalfa.

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